Reference: *Biol. Bull.* **180:** 119–124. (February, 1991)

GABA-Like Immunoreactivity in the Nervous System of *Oikopleura dioica* (Appendicularia)

TOMAS BOLLNER[1,*], JON STORM-MATHISEN[2], AND OLE PETTER OTTERSEN[2]

[1]*Department of Zoology, Stockholm University, S-106 91, Sweden, and* [2]*Anatomical Institute, University of Oslo, Karl Johans gt. 47, N-0162 Oslo 1, Norway*

Abstract. The cellular localization of γ-aminobutyric acid (GABA) has been visualized immunocytochemically in the nervous system of *Oikopleura dioica* by using an antiserum to glutaraldehyde fixation complexes of GABA. The results show GABA-like immunoreactivity in neurons of the brain, in cells of the sensory vesicle, in the caudal ganglion, and in the nerve cord. Positive reactions were also found at the neuromuscular terminals in the tail.

Introduction

Amino acids are considered to be important neurotransmitters in the vertebrate central nervous system (review: Ottersen and Storm-Mathisen, 1984a). Among these, γ-aminobutyric acid (GABA) is a dominant inhibitory neurotransmitter in the brain and spinal cord, and even occurs in the peripheral nervous system (Jessen *et al.*, 1986). GABA has also been reported to be a major inhibitory neurotransmitter in a wide variety of the invertebrate phyla (Gerschenfeld, 1973; Meyer *et al.*, 1986; Vitellaro-Zuccarello and De Biasi, 1988). But no information is available concerning this amino acid in the Urochordata, a group that is often considered to be a phylogenetic link between invertebrates and vertebrates.

The organization of the nervous system of *Oikopleura dioica* has been investigated by several authors (Galt and Mackie, 1971; Holmberg, 1984; Bollner *et al.*, 1986), and the presence of acetylecholinesterase in this species has been reported (Durante, 1959; Flood, 1973). The aim of this investigation was to establish whether *Oikopleura dioica* exhibits GABA immunoreactivity in its central or peripheral neurons.

Received 22 January 1990; accepted 6 November 1990.

* Present address: Department of Biology, University of London, Royal Holloway and Bedford New College, Egham Hill, Egham, Surrey TW20 OEX, UK.

Materials and Methods

Specimens of *O. dioica* Fol, 1872, were collected at the Kristineberg Marine Biological Station and at the Tjärnö Marine Biological Laboratory, both on the west coast of Sweden. For immunocytochemistry the following fixatives were used: (1) 5% glutaraldehyde, (2) 3% glutaraldehyde and 1% paraformaldehyde, or (3) 1% glutaraldehyde and 1% paraformaldehyde, all in 0.1 *M* sodium-phosphate buffer at pH 7.4. After 1 h fixation at room temperature, animals were kept and transported in cold sodium-phosphate buffer with 0.5% glutaraldehyde added. Free floating whole tissues were processed for immunocytochemistry as described by Storm-Mathisen *et al.* (1983) and Dale *et al.*, (1986); the primary anti-serum was diluted 1:300 before processing according to the peroxidase-anti peroxidase technique (Sternberger, 1979).

After fixation in 3% glutaraldehyde and 1% paraformaldehyde or 1% glutaraldehyde alone, both in 0.1 *M* sodium-phosphate buffer at pH 7.4, animals were embedded in Epon resin and cut with a glass knife. One-μm sections were processed on glass slides previously coated with either chrome alum gelatin or poly-L-lysine, and processed by the immunogold-silver (IGS) method, as follows.

The sections were etched for 45 min in sodium-ethanolate, washed 3 × 5 min in absolute alcohol, followed by 2 × 5 min in distilled water, and rinsed briefly in 20 m*M* Tris buffer at pH 7.4 containing 155 m*M* NaCl, 0.1% BSA, and 20 m*M* NaN_3. The same medium was also used for subsequent rinses and for diluting sera. The sections were then incubated with a droplet of 5% normal goat serum in a moist chamber for 20 min. Thereafter they were incubated overnight in 50 μl primary anti-serum diluted 1:100. After a rinse in buffer, followed by washes 3 × 10 min in buffer at pH 8.2, the sections were incubated for 60 min with GAR G5 (goat anti-rabbit immunoglob-

ulin adsorbed to 5 nm colloidal gold, Janssen, Belgium) diluted 1:80 in the same buffer. Finally the sections were washed with the same medium as after primary serum, rinsed in distilled water several times (5 min each at least), developed in 100 μl silver enhancer kit (Janssen), washed in water, and coverslipped.

Indirect immunofluorescense as well as the peroxidase-antiperoxidase (PAP) method of Sternberger (1979) were also tried; the same primary antibodies were visualized by either a second layer of FITC-conjugated sheep anti-rabbit serum (SIGMA), or by unlabeled sheep anti-rabbit serum (Statens Bakteriologiska Laboratorium, Stockholm) followed by PAP complex (Dakopatts).

The antiserum against glutaraldehyde-conjugated γ-aminobutyric acid (GABA antiserum 26) was raised, purified, and characterized as described previously (Ottersen and Storm-Mathisen, 1984b; Ottersen *et al.*, 1986). For all methods used, the controls included absorption of GABA antiserum with GABA-glutaraldehyde complexes (GABA-G) and glutamate complexes (Glu-G) at final concentrations of 300 μ*M*, or replacement of the primary antiserum with normal rabbit serum. Furthermore, the immunoreactivity of the antiserum used was tested according to the filter disc method described by Ottersen and Storm-Mathisen (1984b). The fixation conjugates spotted on the discs were made from macromolecules extracted from rat brain homogenate and from homogenate of the neural complex from the ascidian *Ciona intestinalis*.

Results

The central nervous system of *Oikopleura dioica* consists of an anterior ganglion (brain) and tail ganglia. The anterior part of the brain is extended into paired bulbs, and in the mid-region it has a sensory vesicle. The brain is connected to several ganglia in the tail by a solid nerve cord. The largest of these tail ganglia is referred to as the caudal ganglion (Figs. 1, 2).

Although the brain is hard to see in whole mounts due to the thick oikoplast epithelium, GABA-like immunoreaction was observed in the paired anterior bulbs described by Bollner *et al.*, (1986). In semithin sections, a positive reaction is easily seen in some of the neurons in the bulbs (Fig. 3). In the rest of the brain, staining with the GABA anti-serum were found: in one cell located ventrally in the mid region, in one of the most caudal cells (Figs. 2, 4), and in a dorsal cell close to the sensory vesicle (Fig. 5). Furthermore, immunopositive staining was seen in the epithelial cells referred to as the "brain vesicle cells" by Holmberg (1984) (Fig. 6).

Two immunostained cell bodies situated in the caudal part of the caudal ganglion were observed in semithin sections (Fig. 7) and in the whole mount preparations (Fig. 8). Immunoreactive fibers were seen in both semithin

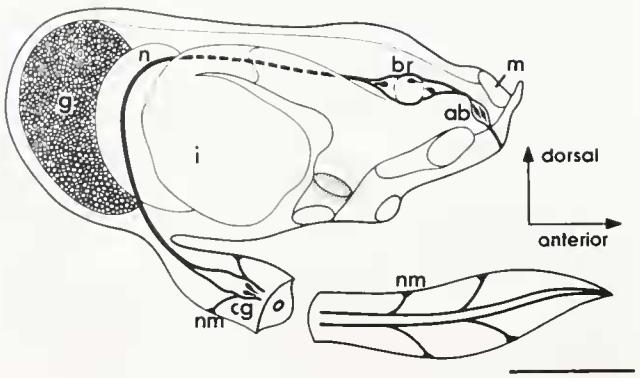

Figure 1. Schematic drawing of *Oikopleura dioica* showing the position of the nervous system with the immuno-positive neurons indicated, the position of the sensory vesicle is indicated by the dotted circle. ab, anterior bulb; br, brain; cg, caudal ganglion; g, gonads; i, intestine; m, mouth; n, nerve cord; nm, neuromuscular junction. Bar = 200 μm.

sections (Fig. 7) and in whole mounts. Transverse semithin sections through the tail showed GABA-like immunoreactivity in the large nerve terminals (Fig. 9) innervating the muscles (*cf.* Flood, 1973, 1975) and in fibers of the dorsal nerve cord. Furthermore, positive staining was often seen in the epithelial cells of the notochord. Because the tail of the animal is twisted 90° counter-clockwise, the dorsal nerve cord is seen to the right of the notochord in a frontal view of a transverse section of tail.

The semithin sections from animals fixed with 1% glutaraldehyde processed using the immunogold technique gave a stronger reaction than any other method. The PAP-method showed weak staining in the anterior bulbs and in the caudal ganglion, whereas the FITC-incubated sections showed clear label in the same regions as did the immunogold method. However, all methods showed similar patterns of immunoreactivity.

The anti GABA serum, either absorbed with glutamate-glutaraldehyde complex (Glu-G) or not, produced selective staining of the GABA conjugates on the filter discs, but no significant staining could be seen after pretreatment of the GABA antiserum with GABA-glutaraldehyde complex (GABA-G) (Fig. 8). Similar results were obtained with spots of amino acids conjugated to macromolecules from rat and *Ciona*, suggesting that the previously demonstrated specificity is valid also for urochordates. In whole mounts as well as in semithin sections, the reaction was virtually abolished when antisera treated with GABA-G or normal rabbit serum were used instead of GABA antiserum. Treatment of the anti serum with Glu-G did not have this effect.

Discussion

Many investigations have established the presence of either GABA or glutamic acid decarboxylase (GAD),

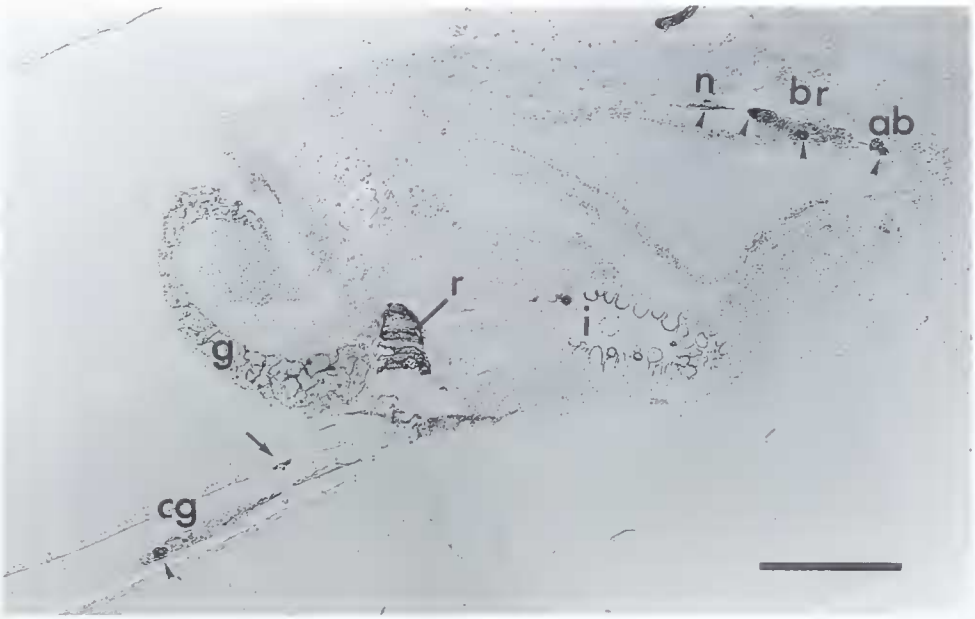

Figure 2. Sagittal section through the whole animal showing the localization and GABA-like immunoreactivity (arrowheads) of the brain (br), one of the anterior bulbs (ab), the nerve cord (n), the caudal ganglion (cg) and in a neuromuscular junction of the tail (arrow). Staining can also be seen in the gonads (g), at the apical surface of some of the intestinal cells (i), and in the rectum (r). No staining could be seen in an adjacent section treated with anti-GABA/GABA-G. IGS method. Differential interference contrast. Bar = 100 μm.

which catalyzes the synthesis of GABA from glutamate, in lower chordates and in invertebrates (Osborne, 1972; Osborne *et al.*, 1979; De Biasi, 1986). More recently, the use of specific antibodies (Storm-Mathisen *et al.*, 1983) has led to a more precise knowledge about the cellular localization of GABA in both vertebrates (Ottersen and Storm-Mathisen 1984, a, b; Roberts *et al.*, 1987) and invertebrates (Bicker *et al.*, 1985; Meyer *et al.*, 1986; Homberg *et al.*, 1987). This is, to our knowledge, the first immunocytochemical study on the occurrence of amino acids in the nervous system of a protochordate. Using biochemical analyses, Osborne *et al.* (1979) found GABA and several other putative amino acid neurotransmitters in homogenates of the cerebral ganglion of another protochordate, the tunicate *Ciona intestinalis*.

The present investigation shows the cellular localization of a GABA-like substance in the nervous tissue of *O. dioica*. The GABA-positive cells in the anterior bulbs and in the rest of the brain are thought to be neurons judging from their location and their ultrastructural appearance previously described by Bollner *et al.*, (1986, unpubl.). Also, most of the cells in the caudal ganglion are considered to be neurons. However, one of its anterior cells, a large ependymal cell, produces the Reissner's fiber (Holmberg and Olsson, 1984). The significance of GABA-like immunoreactivity in the neurons of the central nervous system is difficult to evaluate. Although they may be neurons with inhibitory functions, it should be remembered that GABA may also have depolarizing effects (Alger and Nicoll, 1982). In addition to the neural localization, GABA was clearly present in epithelial cells. This agrees with the situation in vertebrates, where GABA has been demonstrated in non-neural epithelial cells (Orensanz *et al.*, 1986; Davanger *et al.*, 1989). Amino acids in general are also known to modulate osmoregulation (see Gilles, 1979, for review). Synthesis of GABA has been reported to occur in fish erythrocytes where it may participate in the maintenance of a constant cell volume (Fugelli *et al.*, 1970). *O. dioica* is an isosmotic animal, and the vesicle and the chorda are the only internal structures not totally surrounded by hemolymph, and therefore might use GABA for regulating the intracellular osmolarity.

The muscles in the tail are innervated both by fibers branching directly from the nerve cord and from perikarya along the cord (Flood, 1973). These nerves have elaborate end-arborizations on the surface of the muscle cells and are thought to be cholinergic (Flood, 1975; Bone and Mackie, 1982). Cholinergic neuromuscular transmission is widely distributed throughout the animal kingdom, but GABA-ergic inhibition of muscles is only known in invertebrate phyla (Gerschenfeld, 1973). GABA-like immunoreactivity has been demonstrated in inhibitory nerves in insect muscle (Bicker *et al.*, 1988; Robertson

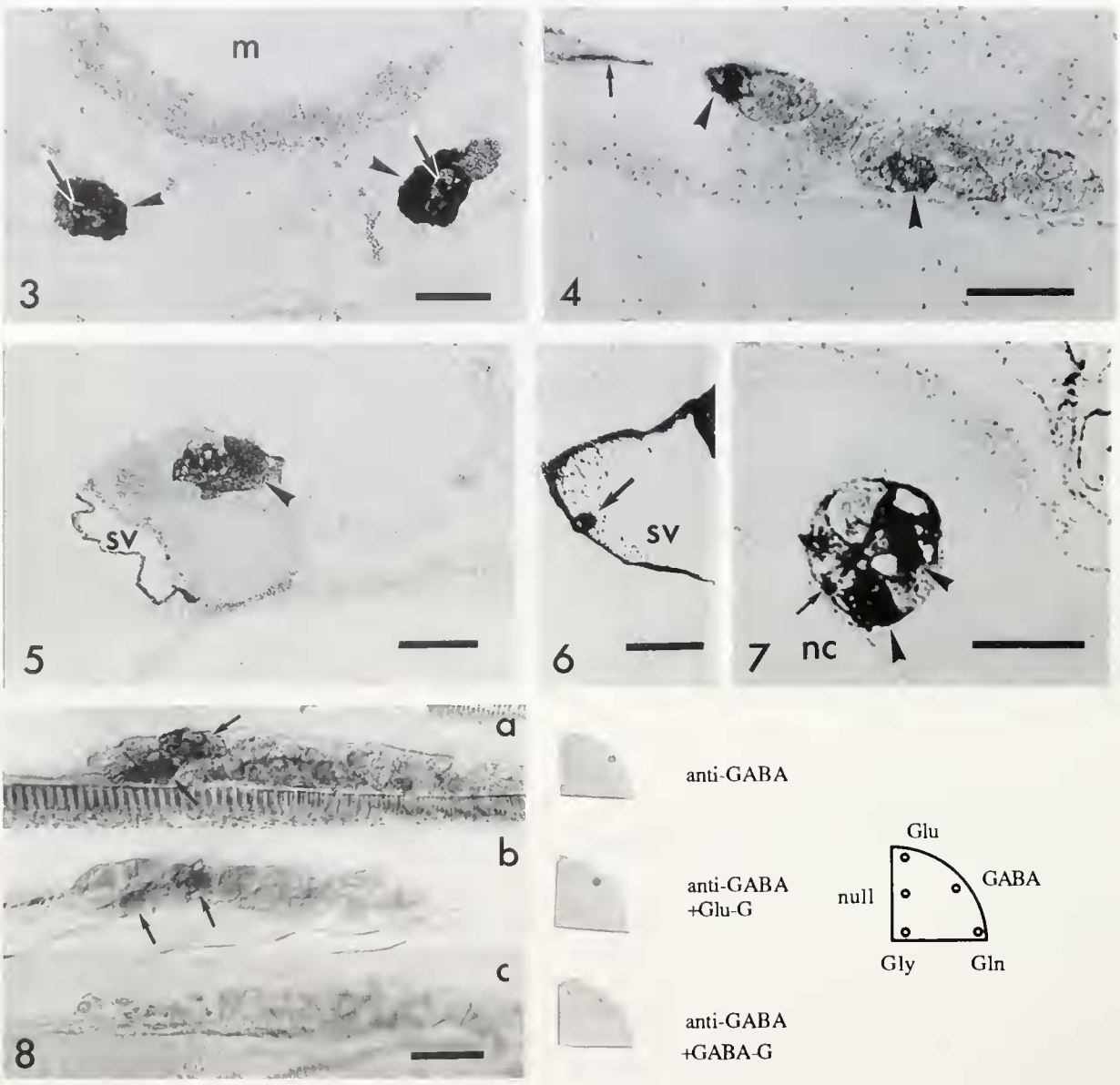

Figure 3. Transverse section through the anterior bulbs with staining in neurons (arrowheads) and in some of the fibers of the neuropile (arrows). m, mouth. IGS method. Differential interference contrast. Bar = 15 μm.

Figure 4. Sagittal section showing positive staining in one cell in the mid part and in one cell in the rear part of the brain (arrowheads) and also in a fiber of the nerve cord (arrow). IGS method. Differential interference contrast. Bar = 15 μm.

Figure 5. Transverse section through the brain in the region of the sensory vesicle (sv), with staining in one cell body (arrowhead). IGS method. Differential interference contrast. Bar = 15 μm.

Figure 6. Section through the sensory vesicle (sv) showing positive staining in one of the brain vesicle cells (arrowhead) and in the epithelial cells forming the vesicle wall. IGS method. Differential interference contrast. Bar = 15 μm.

Figure 7. Transverse section through the caudal ganglion showing positive staining in cell somata (arrowheads) and in neuropile fibers (arrow); nc, notochord. IGS method. Differential interference contrast. Bar = 10 μm.

Figure 8. Whole-mount preparations of the caudal ganglion showing positive reaction in neurons (*arrows*) after treatment with anti-GABA (a) and anti-GABA/Glu-G (b) and no reaction after treatment with anti-GABA/GABA-G (c), corresponding control filter discs with amino acids conjugated to macromolecules from *Ciona* neural tissue by glutaraldehyde are shown at the right. Gln, glutamine; Gly, glycine; null, glutaraldehyde-treated protein with no amino acid added. PAP method. Bar = 15 μm.

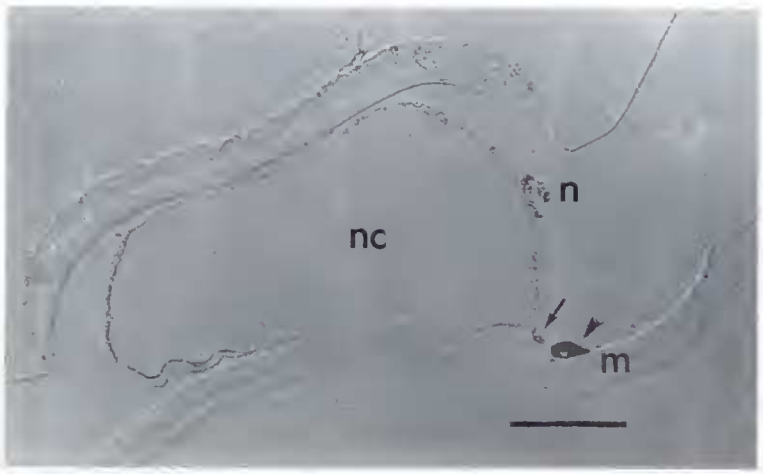

Figure 9. Transverse section through the tail near the trunk, frontal view. GABA-like immunoreaction can be seen in a neuromuscular terminal (arrowhead) and in the nerve cord (n). This section also shows staining in a part of the fiber between the nerve cord and the neuromuscular terminal (arrow). m, muscle; nc, notochord. IGS method. Differential interference contrast. Bar = 20 μm.

and Wisniowski, 1988). Our finding that neuromuscular synapses of *O. dioica* show GABA-like immunoreactivity is the first indication that GABA may also act as neuromuscular inhibitory substance in some chordates.

More work is needed to establish whether GABA is a neurotransmitter in *O. dioica*. Its possible role in osmoregulation in the vesicle should also be subject to further investigation.

Acknowledgments

We are grateful to A. T. Bore and Y. Lilliemark for technical assistance. This investigation was supported by the Hierta-Retzius and Lars Hiertas Minne foundations.

Literature Cited

Alger, B. E., and R. A. Nicoll. 1982. Pharmacological evidence for two kinds of GABA receptor on rat hippocampal pyramidal cells studied *in vitro*. *J. Physiol.* **328:** 125–141.

Bicker, G., S. Schäfer, and T. G. Kingan. 1985. Mushroom body feedback inter neurons in the honeybee show GABA-like immunoreactivity. *Brain Res.* **360:** 394–397.

Bicker, G., S. Schäfer, O. P. Ottersen, and J. Storm-Mathisen. 1988. Glutamate-like immunoreactivity in identified neuronal populations of insect nervous systems. *J. Neurosci.* **8:** 2108–2122.

Bollner, T., K. Holmberg, and R. Olsson. 1986. A rostral sensory mechanism in *Oikopleura dioica* (Appendicularia). *Acta Zool.* **67:** 235–241.

Bone, Q., and G. O. Mackie, 1982. Urochordata. Pp. 473–535 in *Electrical Conduction and Behavior in "Simple" Invertebrates*, G. A. B. Shelton, ed. Oxford University Press, Oxford.

Dale, N., O. P. Ottersen, A. Roberts, and J. Storm-Mathisen. 1986. Inhibitory neurons of a motor pattern generator in *Xenopus* revealed by antibodies to glycine. *Nature* **324:** 255–257.

Davanger, S., O. P. Ottersen, and J. Storm-Mathisen. 1989. GABA immunoreactive cells in the rat gastrointestinal epithelium. *Anat. Embryol.* **179:** 221–226.

De Biasi, S., C. Frassoni, and L. Vitellaro Zuccarello. 1986. Glutamic acid decarboxylase (GAD)-like immunoreactivity in the pedal ganglion of *Mytilus galloprovincialis*. *Cell Tissue Res.* **244:** 591–593.

Durante, M. 1959. Sulla localizzazione istochimica della acetilcolinesterasi lungo lo sviluppo di alcune ascidie e in appendicularie. *Acta Embryol. Morphol. Exp.* **2:** 234–243.

Flood, P. R. 1973. Ultrastructural and cytochemical studies on the muscle innervation in Appendicularia, Tunicata. *J. Microsc.* **18:** 317–326.

Flood, P. R. 1975. Scanning electron microscope observations on the muscle innervation of *Oikopleura dioica* Fol (Appendicularia, Tunicata) with notes on the arrangement of connective tissue fibers. *Cell Tissue Res.* **164:** 357–369.

Fol, H. 1872. Etudes sur les appendiculaires du détroit de Messine. *Mém. Soc. Phys. Hist. Nat. Geneve* **21:** 445–449.

Fugelli, K., J. Storm-Mathisen, and F. Fonnum. 1970. Synthesis of γ-aminobutyric acid in fish erythrocytes. *Nature* **228:** 1001.

Galt, C. P., and G. O. Mackie. 1971. Electrical correlates of ciliary reversal in *Oikopleura*. *J. Exp. Biol.* **55:** 205–212.

Gerschenfeld, H. M. 1973. Chemical transmission in invertebrate central nervous system and neuromuscular junctions. *Physiol. Rev.* **53:** 1–119.

Gilles, R. 1979. Intracellular organic osmotic effectors. Pp. 11–154 in *Mechanisms of Osmoregulation in Animals*, R. Gilles, ed. Wiley Chichester, New York.

Holmberg, K. 1984. A transmission electron microscopic investigation of the sensory vesicle in the brain of *Oikopleura dioica* (Appendicularia). *Zoomorphology* **104:** 298–303.

Holmberg, K., and R. Olsson. 1984. The origin of Reissner's fibre in the appendicularian, *Oikopleura dioica*. *Viddensk. Medd. Dan. Naturhist. Foren.* **145:** 43–52.

Homberg, U., T. G. Kingan, and J. G. Hildebrand. 1987. Immunocytochemistry of GABA in the brain and suboesophageal ganglion of *Manduca sexta*. *Cell Tissue Res.* **248:** 1–24.

Jessen, K. R., J. M. Hills, and J. Saffrey. 1986. Immunohistochemical demonstration of GABA ergeic neurons in the enteric nervous system. *J. Neurosci.* **6:** 1628–1634.

Meyer, E. P., C. Matute, P. Streit, and D. R. Nässel. 1986. Insect optic lobe neurons identifiable with monoclonal antibodies to GABA. *Histochemistry* **84:** 207–216.

Orensanz, L. M., I. Fernández, R. Martín del Río, and J. Storm-Mathisen. 1986. Gamma-aminobutyric acid in the rat oviduct. *Adv Biochem. Psychopharmacol* **42:** 265–274.

Osborne, N. N. 1972. Occurrence of glycine and glutamic acid in the nervous system of two fish species and some invertebrates. *Comp. Biochem. Physiol.* **43B:** 579–585.

Osborne, N. N., V. Neuhoff, E. Ewers, and H. A. Robertson. 1979. Putative neurotransmitters in the cerebral ganglia of the tunicate *Ciona intestinalis*. *Comp. Biochem. Physiol.* **63C:** 209–213.

Ottersen, O. P., and J. Storm-Mathisen. 1984a. Neurons containing or accumulating transmitter amino acids. Pp. 141–146 in *Classical Transmitters and Transmitter Receptors in the CNS Part II.* A. Björklund, T. Hökfelt, and M. J. Kuhar, eds. Handbook of Chemical Neuroanatomy, Vol. 3 Elsevier, Amsterdam.

Ottersen, O. P., and J. Storm-Mathisen. 1984b. Glutamate- and GABA-containing neurons in the mouse and rat brain, as demonstrated with a new immunocytochemical technique. *J. Comp. Neurol.* **229:** 374–392.

Ottersen, O. P., J. Storm-Mathisen, S. Madsen, S. Skumlien, and J. Strømhaug. 1986. Evaluation of the immunocytochemical method for amino acids. *Med. Biol.* **64:** 147–158.

Roberts, A., N. Dale, O. P. Ottersen, and J. Storm-Mathisen. 1987. The early development of neurons with GABA immunoreactivity in CNS of *Xenopus laevis* embryos. *J. Comp. Neurol.* **261:** 435–449.

Robertson, R. M., and L. Wisniowski. 1988. GABA-like immunoreactivity of identified inter neurons in the flight system of the locust, *Locusta migratoria*. *Cell Tissue Res.* **254:** 331–340.

Sternberger, L. A. 1979. *Immunocytochemistry,* Second Edition. John Wiley and Sons, New York, Chichester.

Storm-Mathisen, J., A. K. Leknes, A. T. Bore, J. L. Vaaland, P. Edminson, F-M. S. Haug, and O. P. Ottersen. 1983. First visualization of glutamate and GABA in neurons by immunocytochemistry. *Nature* **301:** 517–520.

Vitellaro Zuccarello, L., and S. De Biasi. 1988. GABA-like immunoreactivity in the pedal ganglia of *Mytilus galloprovincialis*: light and electron microscopic study. *J. Comp. Neurol.* **267:** 516–524.